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Cooling-Induced Activation of the Pericardial Organs of the Spiny Lobster, *Panulirus japonicus*

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Abstract. Ligamental nerves, extensions of the pericardial neurohemal organ of the spiny lobster, produce compound action potentials during cooling of the body, and become silent with warming. Heart-activators released from the pericardial organs into perfusate were collected from the antennule and leg stumps. The perfusate samples were bioassayed using the isolated heart and cardioarterial valves. The extent of heart activation was greatest in samples obtained during the first phase of cooling and was lowest during the initial phase of rewarming. The levels of cardioexcitor substances were clearly related to the firing behavior of the ligamental nerves. Moreover, one of the active factors produced responses identical to octopamine, a known pericardial organ amine. It is proposed that octopamine is released from the ligamental nerve terminals into the blood during cooling of the body.

Introduction

Alexandrowicz (1953) found neurohemal structures in the crustacean pericardium and called them pericardial organs. Since then, cardioactive substances of the organs have been examined using several decapod species (Welsh, 1961; Cooke and Sullivan, 1982). Dopamine and 5-hydroxytryptamine (serotonin) were identified first as pericardial amines (Florey and Florey, 1954; Cooke and Goldstone, 1970). Recently, proctolin, CCAP, and FMRFamide-related peptides were identified in the decapod pericardial organs (Sullivan, 1979; Kobierski *et al.*, 1987; Stangier *et al.*, 1987; Timmer *et al.*, 1987; Dircksen and Keller, 1988). The spiny lobster *Panulirus interruptus* has octopamine, serotonin, and proctolin but little dopamine in the pericardial organs (Sullivan *et al.*, 1977). These hormones are released by electrical stimulation of the organs (Cooke, 1964; Sullivan, 1978; Berlind and Cooke, 1970).

Serotonin activates the small cardiac neurons of decapods (Cooke and Hartline, 1975; Kuramoto and Ebara, 1988), whereas octopamine inhibits them (Benson, 1984) and directly enhances the cardiac muscle contraction (Kuramoto and Ebara, 1991). Thus it has been suggested that serotonin and octopamine exert antagonistic effects on the first and second systolic contraction (FSC and SSC) in *P. japonicus* (Kuramoto and Ebara, 1991). The pericardial peptides all may have excitatory actions on the heartbeat (Sullivan and Miller, 1984; Wilkens and McMahon, 1992; Yazawa and Kuwasawa, 1992). The flap muscles of cardioarterial valves regulate the cardiac outputs in crustaceans (Kuramoto and Ebara, 1984b; 1989; Kihara and Kuwasawa, 1984; Kihara *et al.*, 1985; Fujiwara-Tsukamoto *et al.*, 1992). The pericardial hormones modulate the flap muscle tension (Kuramoto and Ebara, 1984b, 1989; Kuramoto *et al.*, 1992). Much evidence suggests that the pericardial hormones exert their actions not only on the cardiovascular system but also on the respiratory, stomatogastric, and locomotor systems (Kravitz, 1988; Harris-Warrick *et al.*, 1989; Kuramoto, 1991; Rajashekhar and Wilkens, 1992). However, no one has yet shown that any natural stimuli result in activation of the pericardial organs. Here we report that the ligamental nerves, extensions of the pericardial organ of the spiny lobster, exhibit an increase in extracellularly recorded propagated electrical activity during a drop in temperature. If cardioactive factors increase in the blood as a result of the cooling-dependent neurosecretion, they can be detected rapidly and simply by our bioassay methods using the isolated heart and cardioarterial valves (Kuramoto and Ebara, 1984b, 1988, 1991).

In this report, we describe the firing properties of pericardial neurosecretory cells in response to cooling, and

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Abbreviations: CCAP, crustacean cardioactive peptide; FSC, first systolic contraction; SSC, second systolic contraction.

demonstrate cooling-dependent increases of cardioactivators in body fluid. A preliminary account of this study has been reported (Kuramoto and Tani, 1991).

Materials and Methods

Japanese spiny lobsters (*Panulirus japonicus*, 200 g wt) were used for obtaining body fluid ($n = 10$) and for bioassay ($n = 10$). An antennule and a fifth leg of the animal were cut off. Body fluids emanating from the antennule and leg stumps were collected in glass bottles (15 ml) while the physiological saline maintained at room temperature (20 ± 1°C) was injected into the pericardium using a catheter. Because the antennal artery extends straight from the heart to the antennule, the cut antennule provides a direct sampling site for substances secreted into the heart from ligamental nerve terminals. In contrast, the pathway from the heart to the legs is less direct, so perfusate samples from the legs might contain substances released from many neurosecretory sites (Cooke and Sullivan, 1982). The duration sampled by each bottle was 2 or 3 min. To cool the body, the perfusion line was switched by a stopcock to the line for the cold saline (13–15°C). The dead time for flow of the perfusate through the tubing was about 10 s. The duration of cooling experienced by the lobster was 4 or 6 min, and each series of cooling and rewarming was repeated three or four times in each experiment.

Each sample of body fluids (perfusate) was injected by micropipette (1 ml) into the posterior right ostium of an isolated heart from another animal. The perfusate samples were also applied to the anterior and posterior valves (AV, PV) that had been isolated from the lobster heart. Contractions of the heart, AV, and PV were recorded with strain gauges. The recording methods and the characteristics of the heart and valves responding to the pericardial hormones have been reported (Kuramoto and Ebara, 1984a, b, 1988, 1989, 1991).

To record axonal impulses of the ligamental nerves, the pericardium was opened by removing the dorsal part of the thorax (Fig. 1a). To record impulses further from the proximal part of the third segmental nerves (root 3), the cephalic portion and the visceral organs in the thorax were removed ($n = 10$). Nerve impulses were recorded with glass suction electrodes. The pericardium and the thoracic ventral floor were perfused with the warm or cold saline using the perfusion lines described above. Fluid temperature either in the pericardial cavity or on the thoracic floor was monitored with an electronic thermometer using a platinum sensor. The electrical signals were displayed by either a pen recorder (dc—100 Hz) or a thermal dot-array recorder (NEC Sanei Omniace RT2108; dc—50 kHz).

Results

Electrical activity of the ligamental nerves for cooling

The dorsal nerves of the thoracic ganglia (root 3) enter the pericardium, and their branches extend along the three pairs of ligaments. Each of these nerves branches extensively on each ligament. The nerve terminals are distributed near the ostia (Fig. 1a). When the pericardial cavity was perfused with the cold saline, a train of impulses could be recorded from the proximal part of the ligamental nerve (Fig. 1b). The impulses stopped upon rewarming. Delay of the nerve responses from the start of pericardial cooling was about 20 s. The impulse frequency rose to over 5 Hz with the cooling. Large impulses recorded from a ligamental nerve were usually compound. The example shown in Figure 1c was composed of three kinds of impulses as judged by its waveform. Spontaneously generated impulses of the ligamental nerves were often observed at room temperature. However, they ceased with warming of 0.5–2°C (not shown in figure).

Cold stimulation also elicited a few kinds of impulses in the proximal part of root 3 with a delay shorter than 15 s. Figure 2 shows the typical firing pattern of the cool-sensitive neuron. With decreasing temperature (dT > 0.5°C), the cool-sensitive neurons either initiated impulses (Fig. 2a) or raised the frequency of spontaneous impulses (Fig. 2b). The magnitude of the increase in impulse frequency depended on the temperature decrease over a range of 0.5–5.5°C, and impulse frequency increased almost in inverse proportion to the cooling. For this example (Fig. 2b), the maximum rate of cooling was 4°C/min, indicating that the cool-sensitive neuron appeared to adapt slowly to the stimulation. However, the frequency did not always continue to increase with decreasing temperature. In Figure 2a, the response can be seen to plateau 1 min after the onset of stimulation. In contrast, the impulses elicited by cooling stopped quickly with either rewarming or slowing down of cooling rate. Spontaneous impulses of the cool-sensitive neurons also ceased with a small increase in temperature (0.5–2°C). These impulses that responded to cooling and rewarming were usually large in amplitude; most of the small-amplitude impulses were not so sensitive to the cold stimulation.

Bioassay of perfusate samples for cardioactive substances

When isolated hearts were treated with samples of the perfusate collected from cooled animals, the frequency and amplitude of the contractions increased tonically. In contrast, stimulation with perfusate samples collected before and after cooling produced only small, phasic changes in cardiac activity. These findings indicate that cardioac-

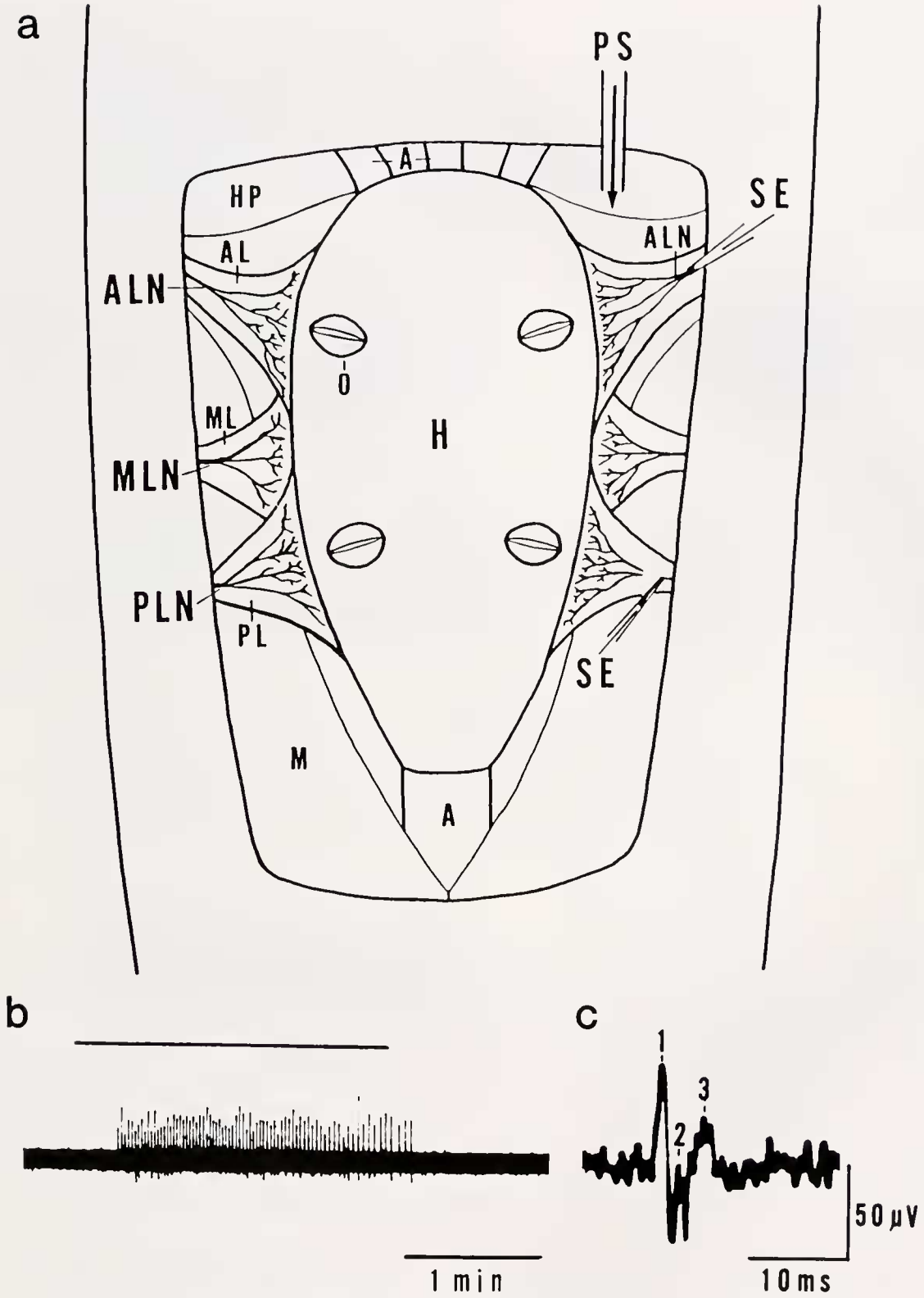

Figure 1. (a) The hepatopancreas (HP) and skeletal muscles (M) surround the heart (H), which is suspended by the anterior, median, and posterior ligaments (AL, ML, and PL). Three anterior and one posterior arteries (A) leave from the heart. Three pairs of ligamental nerves (ALN, MLN, and PLN) branch and terminate on the AL, ML, and PL, respectively, near ostia (O). The pericardial cavity was perfused with the physiological saline (PS) while impulse activity of the ligamental nerves was recorded with the suction electrode (SE). (b) An extracellular recording of electrical activity in an ALN in response to pericardial cooling (dT = 3°C). The duration of cooling is indicated by the bar. (c) The impulse waveform, shown by a fast speed, exhibiting three inflections (1–3).

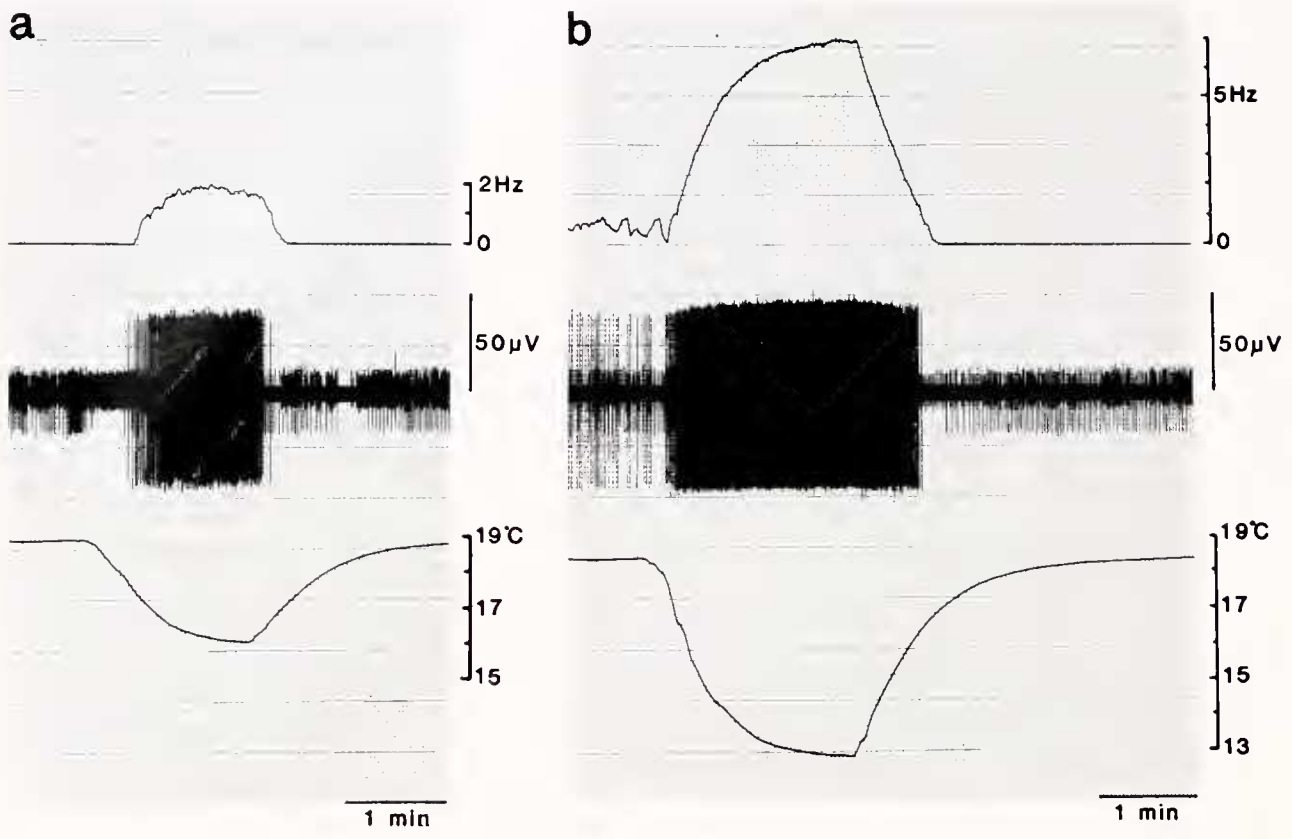

Figure 2. The electrical responses of cool-sensitive neurons in the proximal part of root 3 of the lobster thoracic ganglion. (a) A burst of large-amplitude impulses (middle record) triggered by cooling. The impulse frequency (upper trace) rose to 2 Hz when the temperature (lower trace) dropped from 19 to 16°C but declined during the latter portion of the period of cooling. The spontaneous impulses of small and medium amplitude were unaffected by the change in temperature. (b) Another recording from the same preparation as (a). The impulse frequency (upper trace) rose from 0.5 to 7 Hz in response to cooling (lower trace) from 18.5 to 13°C. Note that the increase in impulse frequency is closely related to the decrease in temperature, since the cooling rate (4°C/min) is higher than the rate at which spike adaptation occurs.

tivators are present in body fluids during periods of internal cooling.

Since the pericardial hormones octopamine and serotonin have differential effects on the FSC and the SSC, the cardiac responses to applied perfusate samples were scrutinized to identify selective differences in FSC or SSC activity. Figure 3 shows an example of the bioassays using a heart whose beat is deficient in the SSC (Kuramoto and Ebara, 1984a, 1988). The perfusate samples obtained before and after cooling had a slight action on the frequency of the SSC. In contrast, the perfusate samples collected during cooling enhanced the FSC over a 5-min period and increased the SSC for 2 min. These effects on the FSC and the SSC will be discussed later in connection with identification of cardioactivators contained in the sample.

The posterior cardioarterial valve (PV) contracted in response to samples of perfusates. The contractile activity was greatest during the first phase of cooling (C1) and lowest during the initial phase of rewarming (W1) (Fig. 4 a–d). The highest and lowest activities of these valves were comparable to those generated by 10^{-7}–10^{-6} M and 10^{-8} M or less octopamine, respectively (Fig. 4e, f).

The cardioactive factors in the perfusate were further examined using both the AV and PV. The largest responses were produced by body fluid sampled during the first phase of cooling (C1). Figure 5 shows an example of the positive results (10 from 16 trials). Active factors in the samples caused the AV to relax and the PV to contract. The relaxation could be observed clearly in the spontaneously contracting anterior cardioarterial valves. One component of the active fluid produced a rapid, short-term (a few minutes) relaxation of the AV, and another component did so slowly over long durations (5–6 min). The effect of octopamine on the AV is identical to the rapid relaxation of AV. Phentolamine (10^{-5} M), an an-

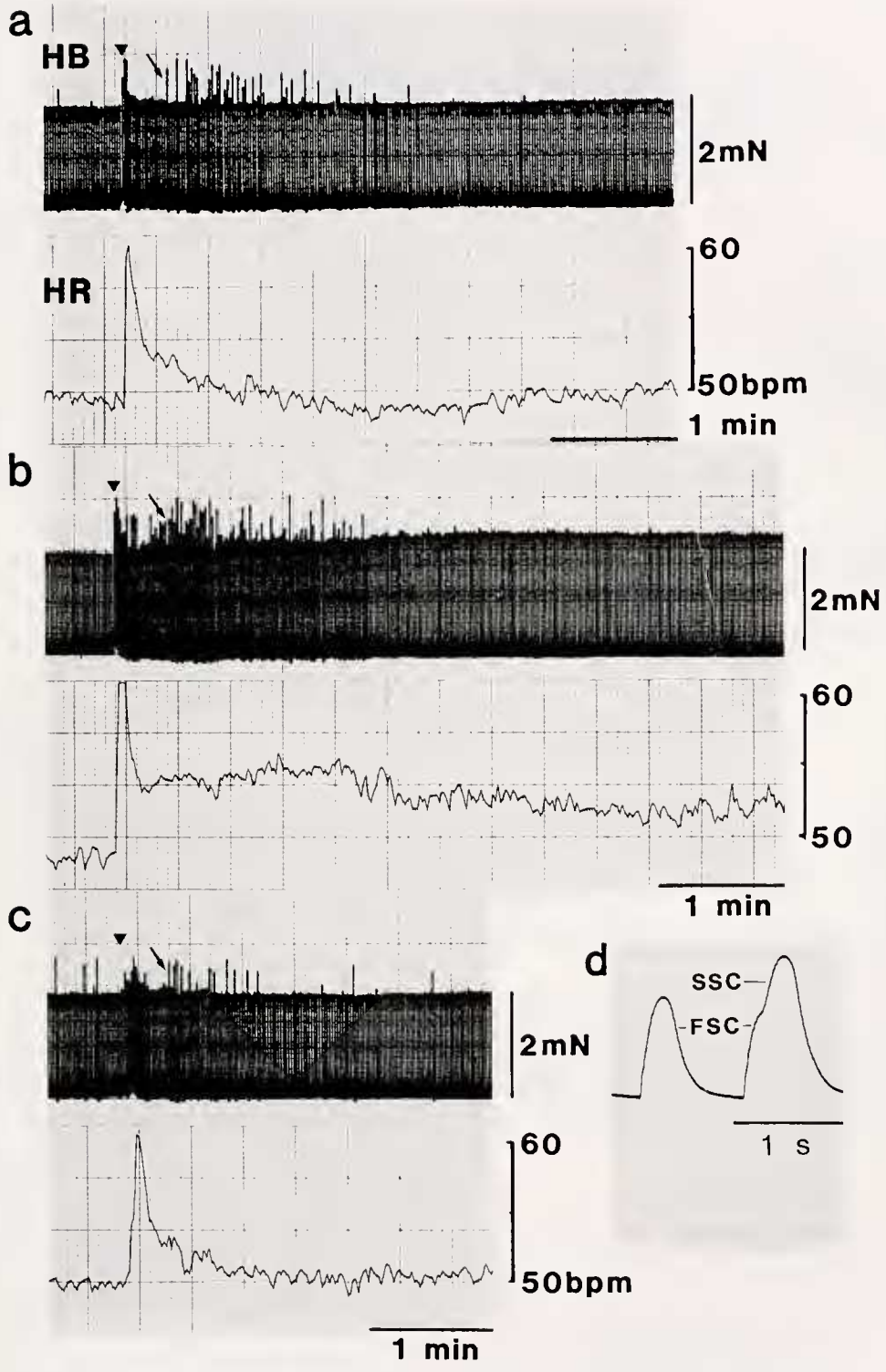

Figure 3. Bioassay for active factors in body fluid using the isolated lobster heart. The body fluid (BF) was collected from the antennule. The heart beat (HB) and rate (HR) were recorded while the perfusate sample (BF, 1 ml) was pulse-applied to the heart at the time indicated by an arrowhead. (a) Response to the BF before cooling. (b) The response to the BF during the first phase of cooling. (c) The response to the BF during the initial phase of rewarming. The rapid increase in beat amplitude (arrows in a–c) is due to the superimposition of SSC on the FSC as shown in (d). The long-lasting and remarkable increases in HR and FSC and the abundant SSC are observed in b.

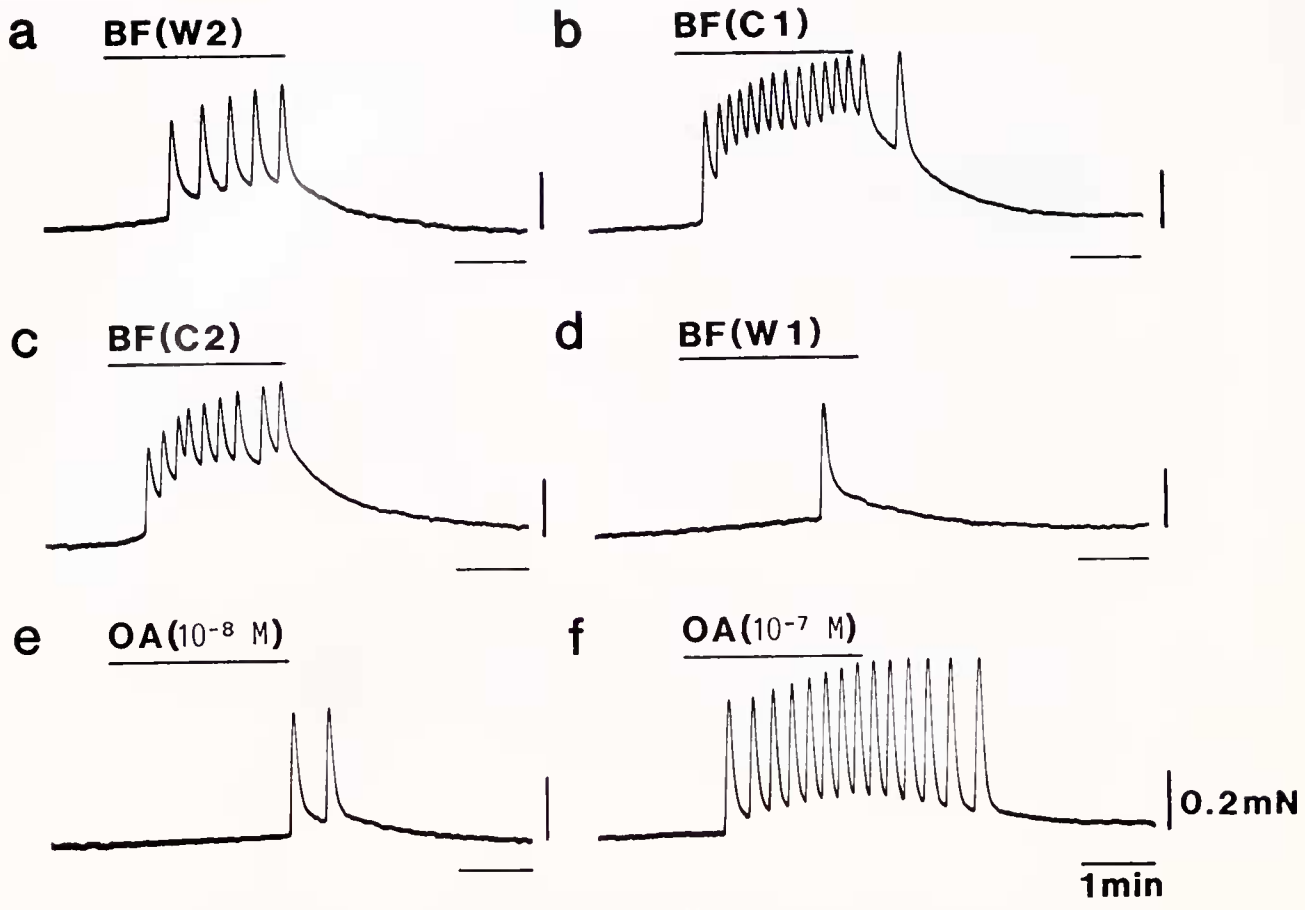

Figure 4. Bioassay of body fluid using the posterior cardioarterial valve of the lobster. The body fluid (BF) was collected from the leg. Each of the perfusate samples (BF, 2 ml) was applied to the valve (bars) while tension of the flap muscle was measured. (a) A typical response of the valve to the BF before cooling (W2). (b) The response to the BF during the first phase of cooling (C1). (c) The response to the BF during the late phase of cooling (C2). (d) The response to the BF during the initial phase of rewarming (W1). (e–f) Two examples of the valve response to octopamine (OA, 2 ml).

tagonist of octopamine, blocked the rapid relaxation of AV (not shown in figure). The relaxing activity of the samples obtained just after the onset of cooling was comparable to that of 10^{-7} *M* octopamine (Fig. 5b, c).

Discussion

The anatomical features of the ligamental nerve plexuses (Fig. 1a) are almost identical with those of *P. interruptus* (Sullivan *et al.*, 1977). Axons in the ligamental nerves come from the thoracic ganglia via root 3. The firing pattern of the ligamental nerves and root 3 in response to cooling was similar (Figs. 1b and 2a). Similar responses of the root 3 have also been observed in the ganglia isolated completely from the thorax of lobsters and shrimps (unpub. data).

The response of the ligamental nerve to cold stimulation occurred following a delay of 20 s. This is at least 5 s longer than the corresponding delay recorded in the proximal part of root 3 (<15 s). However, if the cold receptors were in the ganglia, 5 s might be sufficient to account for the time required for the cold saline perfusing the pericardium to arrive in the thoracic ganglia, and the time for neural conduction from the ganglia to the ligaments under the low temperature. Judging from the cardiac outflow, the latency of 5 s is reasonable (Spaargaren, 1974). The cooling-dependent firing was observed even in the isolated ganglia. Therefore, the impulse activity of the ligamental nerves might be driven by some of the cool-sensitive neurons in the thoracic ganglia.

The amine-containing neurons on thoracic nerve trunks of the lobster *Homarus americanus* extend their axons to the pericardial organs (Evans *et al.*, 1976a, b). However, these neurons fire with increasing temperature (Konishi and Kravitz, 1978). Therefore, they seem to be different from the neurosecretory cells that we found.

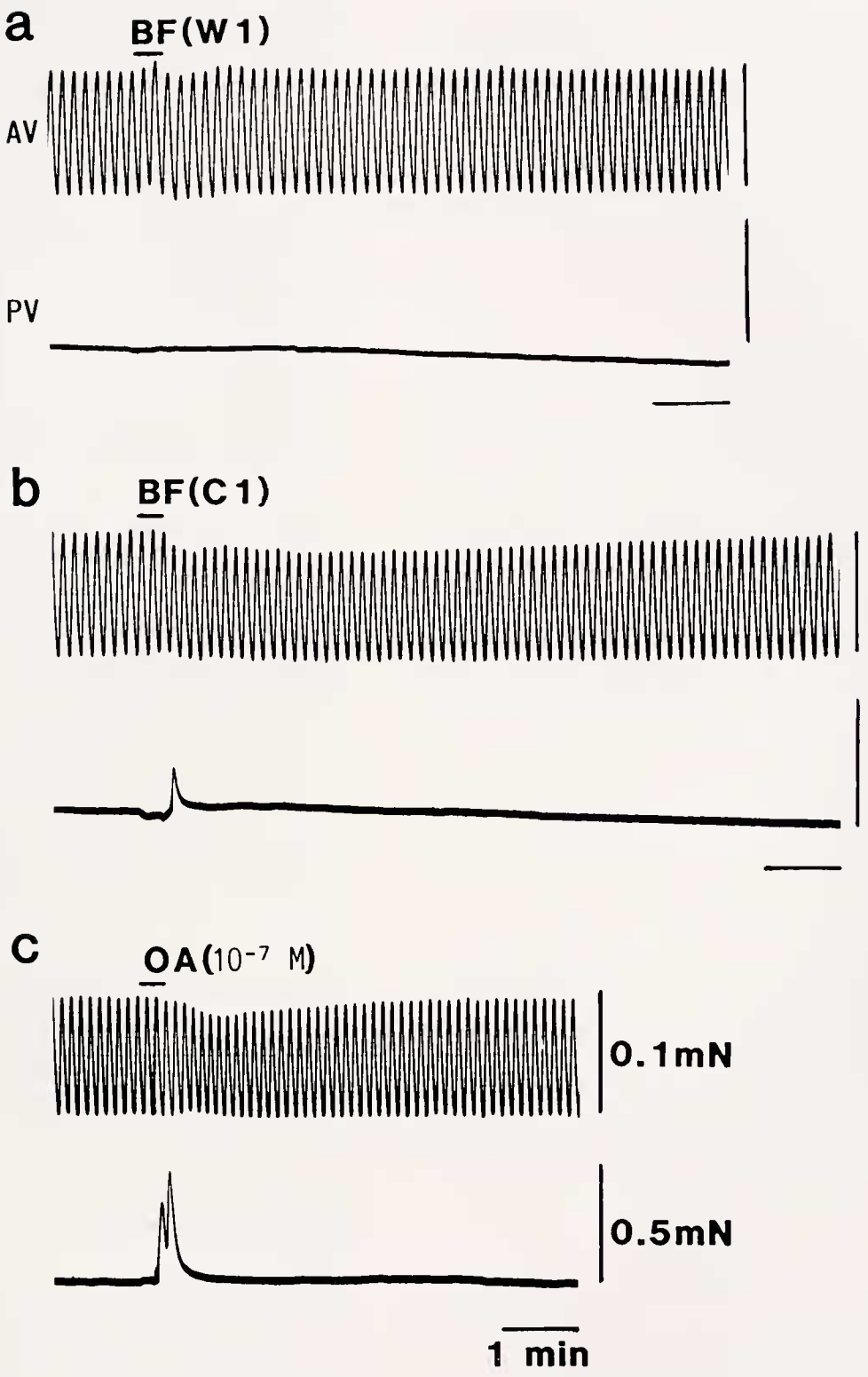

Figure 5. Bioassay of body fluid using the anterior and posterior cardioarterial valves (AV and PV). The body fluid was obtained from the antennule. Each of the perfusate samples (BF, 1 ml) was applied to the valves (bars) while alterations in their tension were recorded simultaneously. (a) Small responses of the AV and PV to the BF before cooling (W1). (b) Differential responses of the AV and PV to the BF during the first phase of cooling (C1). (c) The responses of AV and PV to octopamine (OA). The spontaneously contracting valve (AV) was useful to observe the relaxing effects.

The presence of cool-sensitive neurons in the thoracic and abdominal ganglia and the abdominal stretch receptors of the crayfish has been reported (Kerkut and Taylor, 1956, 1958; Winter, 1973). However, the transduction mechanisms and the physiological role of the neural activity have thus far been unclear. The present study suggests that this cool-sensitivity is probably related to the pericardial neurosecretion. That is, cardioactivators in blood increase with a drop of body temperature, as demonstrated by our bioassay methods (Figs. 3–5). The cardioactivators also may regulate the excitability of nerve and muscle cells.

Long-lasting augmentation of the FSC by octopamine and proctolin has been observed (Kuramoto and Ebara, 1991; unpub. data). Therefore, it seems likely that the tonic increase of FSC observed here (Fig. 3b) was caused by the actions of octopamine or some pericardial peptides present in the perfusate samples.

Serotonin elicits and enhances the SSC by its specific action on the small cardiac neurons (Kuramoto and Ebara, 1988). Therefore, the increase in beat amplitude resulting from the SSC (Fig. 3, arrows) is likely to have been produced by the action of serotonin in the samples.

The responses of the isolated cardioarterial valves provide further insight into the identity of the substances released during cold stimulation. The AV showed rapid and slow relaxations while the PV showed a slight contraction (Fig. 5b). This suggests that the perfusate may contain at least two active factors. Octopamine causes the AV to relax rapidly and the PV to contract, whereas serotonin relaxes both the AV and PV (Kuramoto and Ebara, 1984b). Therefore, the rapid and slow inhibitory factors may be octopamine and serotonin, respectively. Taken together with the sampling conditions, the body fluid sampled from the antennule during cooling probably contains a large amount of octopamine and an undetermined but lesser amount of serotonin. Confirmation of this hypothesis will require biochemical or electrochemical (HPLC) identification of the active factors. Nevertheless, we concluded that the lobster pericardial organs are activated by a decrease in body temperature and release their hormones, one of which is likely to be octopamine.

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